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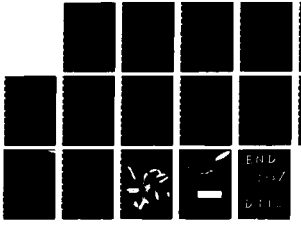

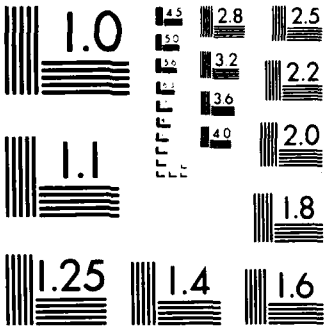

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19. plasmids were not detected by gene hybridization. With respect to the potential for genetic experimentation, most strains grew rapidly (3-4 hr generation time at 30°C), producing colonies on solid media in 2-3 days, The isolated were sensitive to antibiotics used in recombinant DNA experiments and spontaneous drug-resistant mutants were readily selected. Conjugal transfer of plasmids from Escherichia coli to several marine Caulobacters for four broad host-range plasmid incompatability groups was demonstrated, using both self-transmissible plasmids and cloning oriented plasmids which required a helper plasmid. Conjugal transfer of plasmids between freshwater and marine Caulobacters was also demonstrated, in both directions. Native plasmids of approximately 125 kilobase size were found in 2 of the 25 marine Caulobacters strains. These were present in relatively high copy number, appeared stable in laboratory culture and may be useful as stable vehicles of the expression of selected genes. In short, the marine Caulobacters appeared appropriate as candidates for genetic manipulation and the expression of selected genes in the marine environment.

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Marine Caulobacters: Isolation, Characterization and Assessing the Potential for Genetic Experimentation

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Abstract

Twenty-five marine Caulobacters were isolated from littoral marine sources and several aspects of their physiology and morphology were examined, as well as the suitability of these bacteria for genetic manipulation in laboratory cultivation. Caulobacters were readily isolated from all sources, including samples from areas containing pollution-related organic compounds. All isolates grew best in media containing seawater, but about 1/3 would grow if sea salts were replaced with NaCl alone, a few could grow at 1/10 the normal sea salt concentration and one isolate could grow in freshwater media. 48% of the marine isolates and 88% of the freshwater Caulobacters tested grew in anaerobic conditions, indicating that the current categorization of Caulobacters as obligately aerobic bacteria is incorrect. Although some freshwater Caulobacters are able to oxidize manganese, this capability was not found in these marine Caulobacters. 40% of the marine isolates were resistant to mercury chloride concentrations at least ten-fold greater than that tolerated by sensitive bacteria. However a mercury reductase gene comparable to that found in R100-type plasmids was not detected by gene hybridization. With respect to the potential for genetic experimentation, most strains grew rapidly (3-4 hr generation time at 30°C), producing colonies on solid media in 2-3 days. The isolates were sensitive to antibiotics used in recombinant DNA experiments and spontaneous drug-resistant mutants were readily selected. Conjugal transfer of plasmids from Escherichia coli to several marine Caulobacters for four broad host-range plasmid incompatability groups was demonstrated, using both self-transmissable plasmids and cloning-oriented plasmids which require a helper plasmid. Conjugal transfer of plasmids between freshwater and marine Caulobacters was also demonstrated, in both directions. Native plasmids of approximately 125 kilobase size were found in 2 of the 25 marine Caulobacters strains. These were present in relatively high copy number, appeared stable in laboratory culture and may be useful as stable vehicles for the expression of selected genes. In short, the marine Caulobacters appeared appropriate as candidates for genetic manipulation and the expression of selected genes in the marine environment.

Introduction

The Caulobacters are members of the prosthecate or appendaged group of bacteria (32). During their life cycle these bacteria alternate between a monoflagellated, rod-shaped cell (swarmer) and a non-motile cell which has a stalk. The stalk is an elaboration of the bounding cell membranes (36) and also has a quantity of adhesive material (holdfast) at the distal tip which enables the bacterium to attach to surfaces (32). Because of the distinctive changes in morphology and the elaboration of organelles at specific positions on the cell and at predictable times during the life cycle, the bacterium has been studied as a model system to understand differentiation processes in bacteria (34,38). Caulobacter crescentus, a freshwater species, has been used almost exclusively for such studies.

The Caulobacters a are quite ubiquitous in the environment; they can be isolated from soil and freshwater as well as marine and estuarine locations(32). They are well represented in areas of limited nutrients and so have often been categorized as oligotrophic bacteria - those bacteria that prefer and are found in nutrition-poor environments(33). Presumably, the stalked bacteria remain attached to surfaces and survive on nutrients that flow by, occasionally producing swarmer cells as a means of dispersal.

Little is known about marine Caulobacters. Some characterization information for a few isolates was provided in Poindexter's pioneering monograph in 1964 (32), but little related to marine Caulobacters has since been reported. As part of a longer range goal to assess this group of bacteria as part of the microbial fouling community on surfaces in the ocean (12,20), we have begun to isolate and characterize marine Caulobacters. We wish to know how common the Caulobacters are in the marine environment, the fraction of the microbial surface fouling community they represent and whether they are found only in environments with limited nutrition. It is of interest to learn the degree of adaptation to saltwater; typical freshwater Caulobacters are sensitive to elevated ionic strength (J. Smit, N. Agabian, unpublished studies). Other physiological capabilities, such as requirements for oxygen or whether they can fix nitrogen or use inorganic salts as a source of energy are important in evaluating the impact of marine Caulobacters in their environment.

It is also of interest to evaluate whether marine Caulobacters have appropriate characteristics for molecular genetic experimentation. Do they grow on solid media with short enough generation times to make laboratory experiments practical? Can the broad host-range plasmids used in cloning methodologies be introduced by conjugal transfer from Escherichia coli or freshwater Caulobacters? Are there native plasmids that could be developed as stable cloning vectors? We detail in this report our initial work in addressing these questions.

Materials and Methods

Isolation of Marine and Freshwater Caulobacters

The majority of seawater samples were collected from locations in the Puget Sound area of Washington state, including commercial marinas compromised by hydrocarbon and other chemical pollution, sites near storm drain runoff and sites well removed from commercial development. Other samples were taken at sites along the California coast, ranging from Bodega Bay to San Diego. A sampling of seawater off the west coast of India at Goa was also included. A number freshwater Caulobacters were also isolated from tapwater, soil and lakewater sources in Washington state. Water samples were supplemented with peptone to 0.01% and incubated at room temperature for one to two months. When examination of the surface films by light microscopy indicated the presence of a significant number of stalked bacteria, loopfuls of the surface film were diluted appropriately, vigorously vortexed and plated on solid medium. Media contained 2g peptone, 1g yeast extract and 15g agar per liter of seawater (S-PYE) or freshwater (PYE)(32). PYE also contained 0.02% $MgSO_4$. Following incubation at 30°C, individual colonies were examined by light microscopy. Colonies containing cells with appropriate characteristics (motile cells and stalked cells, the latter often attached to one another by the holdfast at the ends of their stalks to form "rosettes") were inoculated into S-PYE or PYE liquid medium and following growth were stored at -70°C after addition of dimethyl sulfoxide to 10%. When more than one isolate was saved from a water sample, their separate identities were confirmed by examination of the protein band patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Other genera of prosthecate bacteria, either Hyphomonas or Hyphomicrobium (7), were commonly seen in surface films of enrichment cultures. In contrast to the Caulobacters, few could be retrieved on solid media.

Physiological Characterization

To evaluate the requirement of marine Caulobacters for sea salts, all isolates were plated on PYE medium, substituting for sea salts concentrations of NaCl, ranging from 0 to 3.5% (w/v). The isolates were also tested for growth on media with varying amounts of commercial preparations of sea salts, ranging from 0 to 3.5% (the latter is a typical salinity of seawater).

Marine strains and freshwater Caulobacter strains were evaluated for growth under anaerobic conditions with the GasPack (BBL-Bectin/Dickison) incubator chamber system. The obligate anaerobe Clostridium sporogenes was grown in the same chambers as a control for anaerobic conditions.

Ability to oxidize manganese was evaluated by growth on S-PYE *media containing* 0.02% $MnSO_4.H_2O$. After 1-2 months of growth, conversion to manganese dioxide was determined by treatment of the plates with benzidine dihydrochloride (which results in a blue color in the presence of manganese dioxide)(22).

Tolerance of Caulobacters to mercury chloride was tested by its addition to S-PYE or PYE media at concentrations up to 30 µg/ml.

Plasmid conjugation

The ability of marine Caulobacters to participate in the conjugal transfer of broad host range plasmids was examined. Plasmids from four incompatability groups (Inc Q, P-1, W and N) known to be conjugally proficient and to replicate in more than one genus of host bacterium (37) were chosen (Table 1). For those that required additional factors in trans for transfer, pRK2013 or pVS109 [a kanamycin sensitive derivative of RP4, (B. Ely and P. Schoenlein, unpublished studies)] were used as helper plasmids in most experiments.

Conjugations were done using standard methods (15). In brief, donor cells (E. coli or another Caulobacter strain) and recipient Caulobacter cells were mixed in appropriate proportions, centrifuged, suspended in a small volume and placed on a 0.22µm cellulose acetate filter on solid medium containing no antibiotics. For E. coli to marine Caulobacter conjugations, the medium was S-PYE. In the case of conjugations between freshwater and marine Caulobacters, PYE medium containing 0.5% sea salts was used. After overnight incubation at 30°C, the cells were suspended and spread on selective antibiotic media. Rifampicin was generally used to select against donor cells, using recipient Caulobacters that had been selected as spontaneously resistant to rifampicin. For some conjugations involving E. coli donor cells and freshwater Caulobacter recipients, trimethoprim was used to select against the donor cells, since freshwater Caulobacters are much less sensitive to the drug (15). This method was not effective in conjugations involving marine Caulobacters. For conjugal transfers between freshwater and marine Caulobacters, the appropriate use of freshwater or saltwater medium was adequate to select against donor cells. All selective antibiotics were used at 50µg/ml, except rifampicin (10 µg/ml) and trimethoprim (150 µg/ml). The use of tetracycline was avoided since its effectiveness was compromised by sea water salts.

Putative transconjugants were confirmed with a colony hybridization method (28), using purified plasmids to make radioactive probes, or when possible, testing for another antibiotic resistance gene known to reside on the plasmid.

Other molecular genetic procedures

For plasmid preparation, the alkaline method was used to purify large quantities of plasmid (8), the boiling method (24) for small quantities and restriction enzyme site mapping, and the method of Kado and Liu (25) was used to search for native plasmids in marine Caulobacters.

Other procedures, such as restriction enzyme digestion, agarose gel electrophoresis, Southern blotting and preparation of radioactive DNA probes by nick translation were done by standard methods (28).

Electron microscopy

Marine bacteria were fixed with glutaraldehyde (2.5% for 1 to 2 hr) before centrifugation and suspension in distilled water. Cells were were examined after negative staining with uranyl acetate or ammonium molybdate.

Results

Isolation of marine Caulobacters

Caulobacters were readily isolated from the surface films of almost all seawater samples taken. This includes samples taken near a storm sewer runoff, where a wide variety of bacteria were noted after addition of the peptone, and samples from within boat marinas, where significant quantities of organic materials and petroleum products were evident. A quantitative analysis of the abundance of Caulobacters, relative to other bacteria, in the various locations was not possible, but there was no obvious increased proportion of Caulobacters in samples taken from areas with lower levels of organic content. Caulobacters were never the numerically dominant morphological type seen; commonly, one of about twenty colonies that grew on solid medium after plating out the surface film of an enrichment culture would be composed of Caulobacters.

If Caulobacters were seen by microscopy in the liquid medium enrichment it was always possible to obtain a Caulobacter from solid medium after plating the culture, suggesting that most marine strains will grow on solid media. This was not the case with the marine Hyphomonas or Hyphomicrobium strains, the other type of stalked bacteria commonly seen. In most cases where they were noted in surface films, they could not be recovered on solid media and those that were recoverable, grew poorly. This characteristic is shared by many marine bacteria (45).

Our results in isolating marine Caulobacters suggested that these strains are not strict oligotrophs, i.e. bacteria that compete effectively only in regions of limited nutrition (33). Caulobacters were found as readily in areas of obvious influx of pollutants and other organic compounds (with a concomitant increase in the number and variety of bacteria noted) as they were in areas well removed human habitation and presumed to be low in available nutrients. However it does appear that Caulobacters are one group of marine bacteria that persist when nutrition becomes limiting. The enrichment method is based on that presumption and indeed the Caulobacters became a significant

fraction of the bacterial population only after lengthy incubation periods. Although these were not quantitative observations, it seems that Caulobacters comprise a small but consistent fraction of the total marine bacterial population throughout coastal waters.

Morphological characteristics

It was often apparent by morphology that the Caulobacter isolates represent distinct populations of stalked bacteria. There was variation in the shape of the cell body, the length of the stalk, the size of the adhesive holdfast and the degree of swarmer cell motility. Most isolates produced rosettes in culture, but the frequency of the rosettes was variable and the number of cells in a rosette varied from only a few to hundreds. Presumably the factors influencing the size of a rosette were the size of the holdfast, length of the stalk and the tendency for the holdfast to stick to itself. The visual indications of strain differences were confirmed by noting different protein patterns by SDS-PAGE and the variation in other physiological characteristics noted below.

Efforts to view the marine strains by negative stain electron microscopy were only partially successful. The bacteria could not be viewed as preparations directly from seawater media because the large amount of salts degrade the image. Most of the strains are seriously disrupted when suspended in distilled water and the use of volatile salts, such as ammonium bicarbonate, did not lessen the damage. Glutaraldehyde fixation preserved the general shape of cells but at times little detail remained or damage related to osmotic changes was still evident. There were exceptions (Fig 1 and 2). In the case of MCS24, the acceptable preservation was probably due to this strain's compatability with freshwater (see below).

Interestingly, cross-walls in the stalk, a common feature in freshwater Caulobacter strains (32), were generally not found in marine strains. The only exception discovered was MCS24 (Fig 1) a strain that was able to grow in freshwater media (see below).

Nutritional and physiological characterization

Nearly all marine Caulobacter strains isolated have proven to be amenable to routine laboratory culture methods. All strains grew well in seawater supplemented with

peptone and yeast extract (S-PYE). Growth rates in S-PYE were determined for representative marine strains. Generation times ranged from 3 to 4 hr at 30°C, which is comparable to most freshwater Caulobacter isolates. Generally, on solid media, colonies of suitable size for most experiments formed within 3 days at 30°C. So far, no strains have been found which will grow significantly in sea water supplemented only with NH_4Cl, phosphate and glucose or maltose.

Bacteria taken from the marine environment are sometimes considered true marine bacteria if there is a demonstrated requirement for sea salts. All the marine isolates were tested for their ability to grow on solid medium containing reduced amounts of sea salts or on a medium with NaCl substituted for sea salts (Table 2). All strains were able to grow on media with commercial preparations of sea salts substituted for sea water. All strains also grew on media containing 1/4 the normal amount of sea salts, but only three of 25 strains were able to grow on 1/10 strength sea salts. In eight of the 25 strains tested, NaCl at 1 or 2% could be substituted for sea salts, although growth was seldom optimal. No strains grew on PYE medium containing 3.5% NaCl.

The freshwater Caulobacter isolates tested thus far have limited tolerance to elevated ionic strength (not shown). In general then, the terrestrial and marine Caulobacters may be physically and possibly genetically separated from one another. However one strain, MCS24, was unique in that it was able to grow in freshwater as well as saltwater. A slower growth rate and irregular morphology in freshwater suggests however that this isolate is much better adapted to marine environments.

All marine Caulobacters and a number of freshwater Caulobacters, both new isolates and laboratory strains, were tested for their ability to grow under anaerobic conditions (Table 3). 48% of the marine strains were able to grow in the same conditions that permitted good growth of Clostridium sporogenes, a bacterium noted for sensitivity to oxygen (ref). In the case of freshwater Caulobacters, the proportion was even greater; 14 of 16 strains (88%) grew under anaerobic conditions. It is likely that amino acids in the growth media served as the fermentable carbon source since the addition of glucose or maltose to the plate media did not significantly stimulate growth.

The marine Caulobacters were also tested for their ability to oxidize manganese as a source of reducing power, as some freshwater Caulobacters are able to do so (22) and this capability occurs commonly in marine bacteria (10,21). None of the isolates were able to produce manganese dioxide from $MnSO_4$ and growth of several strains was inhibited by $MnSO_4$.

A preliminary examination of the ability of marine Caulobacters to fix nitrogen was also made. This was done because several strains in anaerobic conditions appeared to produce colonies on solid media lacking a nitrogen source. All marine strains were examined for the presence of a nitrogenase gene by colony hybridization with the plasmid pSA30, which contains a portion of the *nif* operon of Klebsiella which cross-hybridizes with other characterized nitrogen-fixing bacteria (11). No hybridization was detected in conditions that permitted 30-35% base-pair mismatch.

Tolerance of the toxic metal mercury was examined by growth in the presence of $HgCl_2$ (Table 4). Whereas sensitive strains, both freshwater and marine, tolerate no more than approximately 2 µg/ml of $HgCl_2$, 10 of 23 isolates tested grew with 10 to 20µg/ml present. None grew with 30 µg/ml $HgCl_2$. By comparison, E. coli with plasmid pJP4, which specifies resistance to $HgCl_2$(14), also tolerated no more than 20 µg/ml $HgCl_2$. Typically, resistance to these levels of mercury is associated with the presence of a mercury reductase activity. However, colony hybridization with the plasmid pACNR25, which contains a portion of the R100-type mercury reductase gene (2), in conditions that permit 30-35% base-pair mismatch, did not indicate significant homology in any of the mercury-tolerant strains.

Conjugation proficiency

A number of marine Caulobacters were selected for testing conjugation proficiency with representative plasmids from four incompatability groups known to be conjugative and to replicate in more than one genera of bacteria (Table 1). Plasmids from all the incompatability groups have been introduced into at least one of the marine Caulobacters from donor E. coli (Table 5). Some of the marine Caulobacters have

proven to be more efficient recipients for conjugation than other strains and successful conjugal transfer of a plasmid was readily demonstrated. MCS6 and MCS17 are examples.

Plasmid transfer between freshwater(C. crescentus CB15) and marine Caulobacters (MCS3, MCS6, MCS18) was also demonstrated. RK2 was selected as an example of a large, self-transmissable plasmid. R300B [also known as RSF1010 or R1162 (1,30)] was selected as an example of a smaller conjugal plasmid from another incompatability group which requires factors in trans from a self-transmissable plasmid for transfer between hosts. With both plasmids, conjugal transfer in both directions was demonstrated, indicating that marine Caulobacter strains are not only recipients in conjugal plasmid transfer, but also are able to express plasmid-encoded factors needed to be donors in conjugation.

Native plasmids

All marine Caulobacters were examined for the presence of native plasmids. Plasmids could be demonstrated in only two of the 25 strains, MCS1 and MCS3 (Fig 3). In both cases, the plasmids were large, approximately 125 Kb, and present in large amounts. In MCS3, for example, the plasmid was estimated to average approximately 5 copies per cell.

Discussion

In assessing microbial fouling or attachment to surfaces in the ocean, we have focussed on one bacterial component of the process, the Caulobacters. This genus of bacteria is readily recognized and has a distinctive adhesion organelle for attachment. In addition, if the body of information already available for freshwater Caulobacters is in some ways applicable to marine counterparts, an additional advantage in studying this group of fouling bacteria would be realized. This is especially true with respect to molecular genetic methods, which would be helpful for in-depth analyses of the composition and synthesis regulation of the substances used for attachment and to learn how Caulobacters interact in biofouling communities. Also, it is desirable to develop the ability to introduce and express genes in bacteria that are stable residents of the marine environment, potentially for such things as the production of commercially important compounds, conversion of biomass or degradation of toxic

compounds. As first steps in this study, it was necessary to isolate new strains of marine Caulobacters, begin to accumulate information about their distribution in the marine environment, determine if they are amenable to laboratory culture methods and learn if basic molecular genetic tools can be used with these bacteria.

The procedure for isolation of Caulobacters is not amenable to quantitative studies of the relative abundance of marine Caulobacters. But the observation that they could be readily isolated from nearly any source indicated that they are probably not excluded from most marine environments. For example, exclusion from areas receiving significant input of chemical or organic pollution might be expected for true oligotrophic bacteria.

Many of the marine Caulobacters also may not be excluded from regions that have become anaerobic, such as deeper layers of complex, mature biofouling communities that develop on surfaces. This is the first report that many isolates or species of Caulobacters, both freshwater and marine, are able to grow in anaerobic conditions. Previously, Caulobacters have been described as obligately aerobic organisms (7).

Although Caulobacters persist in areas of limited nutrition, we have not been able to demonstrate that this is due to extraordinary metabolic capabilities, such as nitrogen fixation, growth with only simple sugars or the deriving of energy from inorganic sources such as manganese. The growth noted on nitrogen-deficient medium was likely due to proficient scavenging of minor sources of reduced nitrogen. However the negative results obtained by hybridization probing with a characterized nitrogenase gene may not be definitive. An alternative set of nitrogen fixation genes has been reported for Rhizobium which are induced under conditions of molybdenum limitation (9). A similar pathway may be present in some marine bacteria since sulfate, plentiful in seawater, is an antagonist for molybdenum uptake (P. Bishop, personal communication).

Bacterial resistance to mercury compounds is most often due to a mercury reductase activity which converts Hg^{2+} to Hg^{0} (43). The latter is less toxic and is volatile, eventually leaving the aqueous environment. A significant fraction of the marine Caulobacters were tolerant of elevated mercury levels, yet no hybridization between a

characterized mercury reductase gene and the Caulobacter genomes could be demonstrated. However, there are reports of similar findings with other marine bacteria (27). It may be that in the marine environment, a different class (or classes) of mercury reductase genes is common.

The necessity of seawater for growth of the Caulobacters is some indication of how well adapted these bacteria are to the marine environment. This might be an important consideration since most samples were taken near to shore and the Caulobacters retrieved might have come from terrestrial sources and not be stable members of the the marine microbial community. Most of the isolates did require sea salts and required at least 1/4 the normal concentration. For almost 1/3 of the strains, however, this salt requirement could be met by NaCl alone. Although growth was seldom optimal, the results suggest that these strains needed only elevated levels of sodium ions or a minimum level of total ionic strength. The Caulobacters isolated were not halophiles however, since none grew at 3.5% NaCl concentration.

Of the few isolates that did grow on media with 1/10 normal sea salt concentration, one of them, MCS24, could also grow in freshwater medium, albeit with some difficulty. For another isolate, MCS26, the ability to grow at reduced sea salt concentration may be explainable. This strain was obtained from a sample taken from the Arabian Sea off the west coast of India, where rainwater input from the annual monsoon season lowers the ocean salinity in many areas to about 1% (R. Nagabhushanam, Marathwada University, Aurangabad, India, personal communication). Presumably indigenous bacteria may be able to adapt to such fluxes. MCS24 was the only marine Caulobacter in which stalk crosswalls were found. Whether the presence of crosswalls is directly related to the tolerance of freshwater conditions is unknown. It is also possible that this strain is derived from freshwater stocks, where crosswalls are much more common, while most of the marine strains do not have a terrestrial origin.

The development of molecular genetic approaches to study the Caulobacters or any other group of marine bacteria is at first dependent on being able to use standard laboratory cultivation techniques. The Caulobacter strains isolated all grew on simply-prepared solid media and rapidly enough so that laboratory procedures such as strain purification, conjugation and mutant selection could be readily done. This is in

contrast to many other marine bacteria, such as the Hyphomonas or Hyphomicrobium species discussed above, which are difficult to cultivate on solid media(45). Spontaneous mutation to drug resistance (rifampicin, for example) was readily selected for without the use of mutagenic chemicals. This has not been the case for some other marine bacteria (26).

The demonstration that conjugation was possible with marine Caulobacters using a number of classes of conjugal plasmids was important for future molecular genetic studies with this group of bacteria. A procedure for introduction of plasmid DNA by transformation into Caulobacters, either freshwater or marine, has not been developed. Conjugal transfer of broad host-range plasmids from a donor bacterium is the other general method for introducing foreign DNA into bacteria. The ability to transfer plasmids between freshwater and marine Caulobacters is also significant. The development of molecular genetic approaches and information in freshwater Caulobacters has preceded work with marine strains. Thus, an extensive genetic map has been assembled (3,4), the suitability of several transposons for molecular mutagenesis has been determined (16,17), a recombination-deficient strain has been developed (31) and a transducing bacteriophage is available (6,18). In effect, if a particular genetic manipulation cannot be done directly in marine Caulobacters, often it may be possible to accomplish it in freshwater strains and transfer the result to the marine strains by conjugation.

As alluded to above, one type of desirable molecular genetic experimentation with marine Caulobacters is the expression of foreign genes enabling a variety of useful activities not normally present in the native Caulobacters. But often genes from one organism are not expressed in another. Frequently, the lack of recognition is at the level of transcription promoters. We have the cloned gene for the predominant protein of the a periodic surface structure of C. crescentus, a freshwater strain (40-42). This protein is the most abundant protein in the freshwater strains that express it, apparently due to a strong promoter (J. Fisher, J. Smit and N. Agabian, manuscript in preparation). Preliminary evidence indicates that this promoter is efficiently recognized in several marine Caulobacters (N. Anast and J. Smit, unpublished studies). Thus portions of this gene may be suitable for the development of plasmid expression vectors in both freshwater and marine Caulobacters.

The search for native plasmids was motivated by the interest in developing additional molecular genetic tools for marine Caulobacters. Native plasmids were found infrequently in the marine Caulobacters. A similar observation has been made for freshwater Caulobacters (35). Those found in marine strains were present in relatively high copy number and appeared to be stable in laboratory cultivation. We are currently determining whether these plasmids are transmissable via conjugation and whether they may be suitable as stable vectors for the expression of foreign genes.

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Figure and Table Legends

Figure 1. Negative stain electron microscopy of MCS24. The cells are attached to one another by their holdfasts to form a "rosette", an artifact of high density pure Caulobacter cultures which aids in their identification. The arrows point to stalk crosswalls, which were only found in freshwater Caulobacter strains and this marine strain which can grow in freshwater. Negatively stained with uranyl acetate. Bar indicates 1 µm.

Figure 2. Negative stain electron microscopy of MCS9. Negatively stained with ammonium molybdate. Note that absence of crosswalls in the stalks. Arrow indicates the adhesive holdfast which attracts the stain. Bar indicates 1 µm.

Figure 3. Native plasmids in marine Caulobacters. Plasmid preparations according to the method of Kado and Liu (25) yielded positive results from two strains. a) Size standards (HindIII digest of lambda DNA), b) and c) plasmid preparations from MCS1 and MCS3, respectively.

Table 1. List of plasmids used.

Table 2. Salt requirements for marine Caulobacters.

Table 3. Growth of Caulobacters in anaerobic conditions.

Table 4. Tolerance of mercury chloride by marine Caulobacters.

Table 5. Plasmid conjugation proficiency between *E. coli* and selected marine Caulobacter strains. Conditions for conjugal transfer, including the use of helper plasmids, are described in the text. The "X" indicates a successful conjugal transfer of the plasmid. The absence of an "X" indicates either that transfer of the particular plasmid has so far been unsuccessful or has not been tried.

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Table 1. List of Plasmids Used

Plasmid	Genotype/Characteristics *	Size	Ref.
R300B	Inc Q, Sm^R, $Sulf^R$ mob^+	8.9 Kb	5
pKT230	Inc Q, Sm^R, Km^R mob^+	11.9	1
pGSS33	Inc Q, Ap^R, Tc^R,Cm^R, Sm^R mob^+	13.4	39
RK2	Inc P-1, Ap^R, Km^R, Tc^R mob^+, tra^+	60	29
pRK293	Inc P-1, Km^R, Tc^R mob^+	21.4	13
pVS109	Inc P-1, Ap^R, Tc^R mob^+, tra^+	60	'
pRK2013	Inc P-1, Km^R mob^+	48	19
R46	IncN, Ap^R, Tc^R,Sm^R, $Sulf^R$ mob^+, tra^+	48	23
pSA151	Inc W, Sm^R, Km^R mob^+	13.3	44
pSA30	Tc^R contains portion of *nif* operon	10	11
pACNR25	Tc^R contains portion of R100-type mercury reductase	6.9	2
pJP4	Hg^R, 2,4-dichlorophenoxyacetic acid utilization contains a mercury reductase gene	80	14

'-P. Schoenlein and B. Ely, unpublished studies

*- mob+ indicates the plasmid is capable of conjugal transfer from one host to another.

tra+ indicates that the plasmid has the capacity for self-transfer or can provide necessary factors *in trans* to enable transfer of mob+ plasmids.

Table 2. SALT REQUIREMENTS FOR MARINE CAULOBACTERS

Marine Caulobacter Strain	Growth in 1/10X Sea Salts	Growth in 1or2% NaCl	Growth in Freshwater Medium
MCS1	-	+	-
MCS2	+	-	-
MCS3	-	-	-
MCS4	-	+	-
MCS5	-	-	-
MCS6	-	-	-
MCS7	-	-	-
MCS8	-	-	-
MCS10	-	-	-
MCS11	-	-	-
MCS12	-	-	-
MCS13	-	-	-
MCS14	-	-	-
MCS15	-	+	-
MCS16	-	ND	-
MCS17	-	+	-
MCS18	-	-	-
MCS19	-	-	-
MCS20	-	-	-
MCS21	-	-	-
MCS22	-	-	-
MCS23	-	+	-
MCS24	+	+	+
MCS25	-	+	-
MCS26	+	+	-

Table 3. GROWTH OF CAULOBACTERS IN ANAEROBIC CONDITIONS

Marine Strains		Freshwater Strains	
MCS1	-	*C. crescentus* CB1	+
MCS2	-	*C. crescentus* CB2	+
MCS3	-	*C. crescentus* CB13	+
MCS4	-	*C. crescentus* CB15	+
MCS5	+	*C. bacteriodes*	+
MCS6	+	*C. henrici*	+
MCS7	+	*C. leydii*	+
MCS8	-	*C. subvibroides*	+
MCS10	-	*C. vibroides*	+
MCS11	-	FWC1	+
MCS12	+	FWC2	+
MCS13	+	FWC3	+
MCS14	+	FWC4	-
MCS15	+	FWC5	-
MCS16	-	FWC6	+
MCS17	-	FWC7	+
MCS18	+		
MCS19	-		
MCS20	-		
MCS21	+		
MCS22	-		
MCS23	+		
MCS24	-		
MCS25	+		
MCS26	+		

Table 4. Tolerance of Mercury Chloride by Marine Caulobacters

Strain	Tolerance Concentration
MCS1	≤2 µg/ml
MCS2	≤2
MCS3	20
MCS4	≤2
MCS5	10
MCS6	10
MCS7	≤2
MCS8	≤2
MCS10	20
MCS11	10
MCS12	≤2
MCS13	10
MCS14	10
MCS15	≤2
MCS16	≤2
MCS17	≤2
MCS18	≤2
MCS19	≤2
MCS20	≤2
MCS21	20
MCS22	20
MCS23	10
MCS24	≤2
MCS25	ND
MCS26	ND

Table 5.

PLASMIDS	MARINE CAULOBACTER STRAINS							
	MCS 3	MCS5	MCS6	MCS7	MCS15	MCS17	MCS18	MCS19
Inc Q								
R300B	X	X	X	X	X	X	X	
pKT230		X	X		X	X	X	X
pGSS33			X			X	X	
Inc W								
pSA151						X		X
Inc P-1								
RK2	X		X				X	
pRK293			X	X		X		
Inc N								
R46			X	X		X	X	X

Fig 1

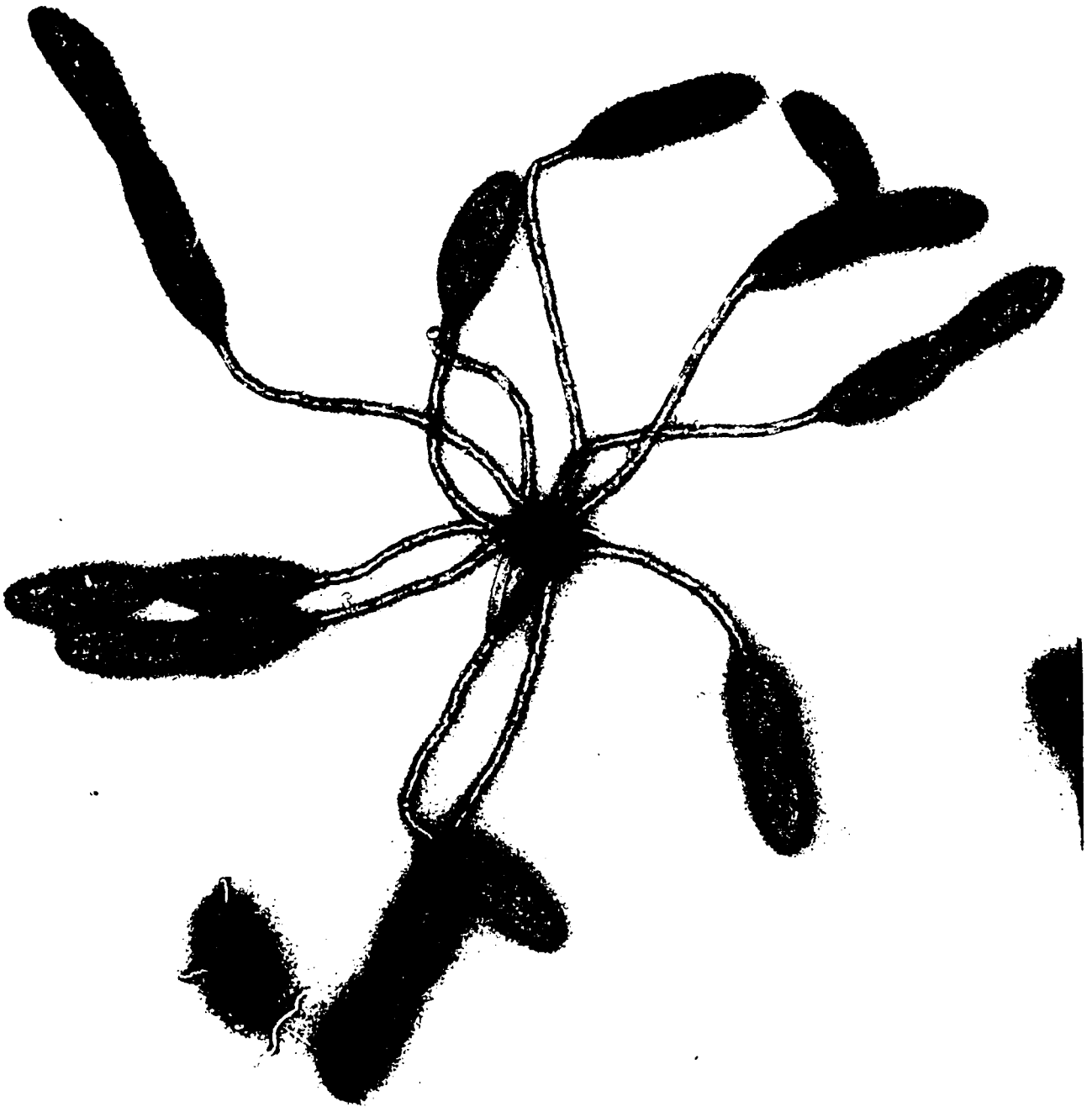
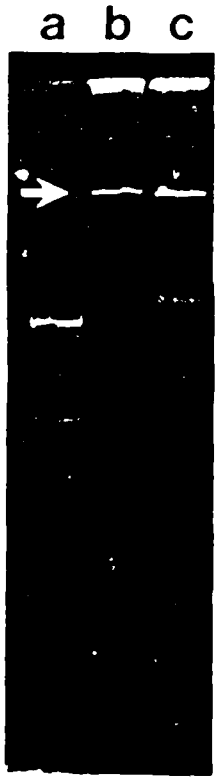
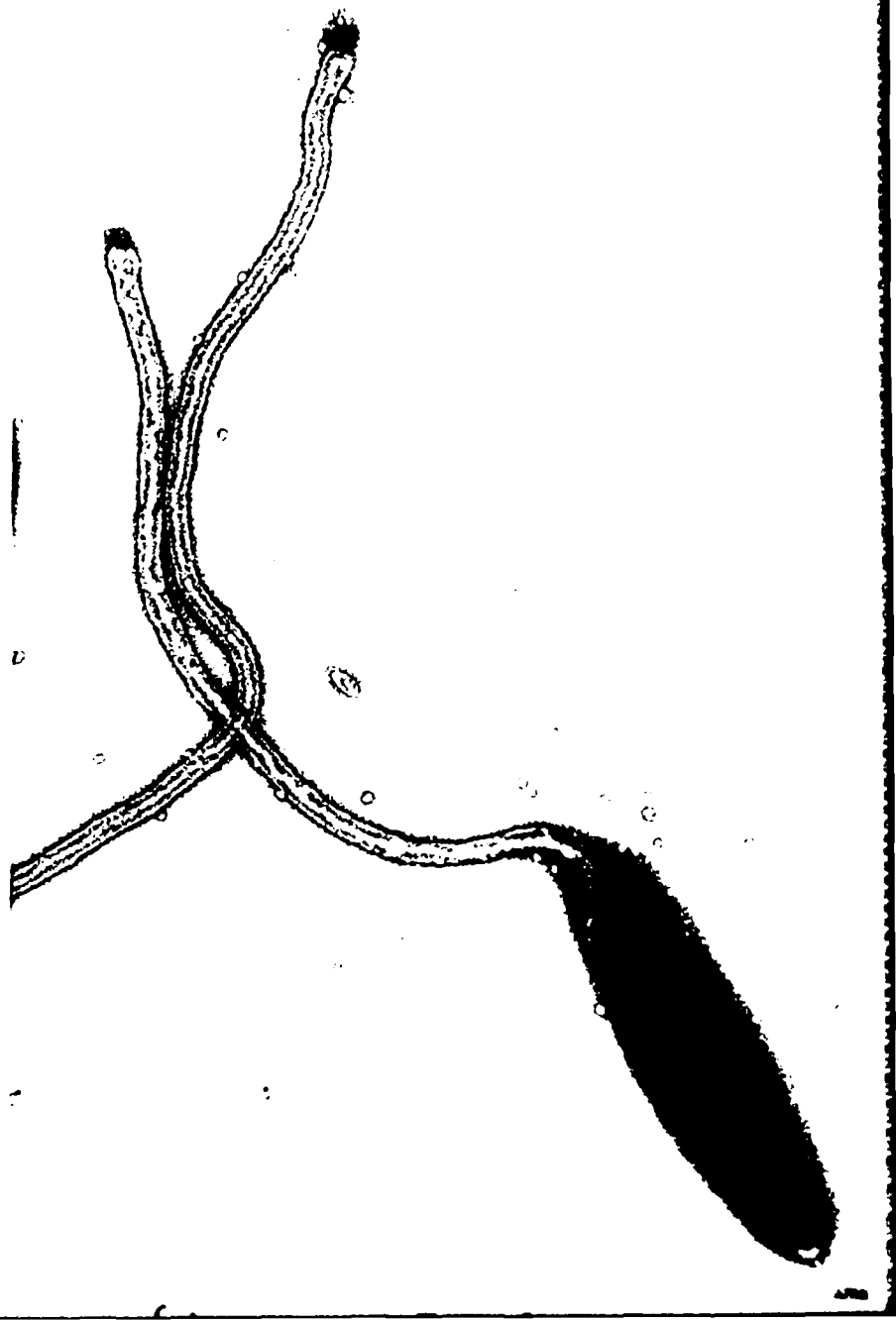


Fig 7





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